Test Methods for Hazard Determination of Metals and Sparingly Soluble Metal Compounds in Soils: Summary from a SETAC Pellston Workshop

Edited by A. Fairbrother on behalf of the SETAC Workshop Steering Committee:

A. Fairbrother, ecological planning and toxicology, inc., USA
P.W. Glazebrook, Rio Tinto Ltd., Australia
N. Van Straalen, Vrije Universiteit, The Netherlands
J.V. Tarazona, INIA, Spain

This summary outlines the Test Methods for Hazard Determination of Metals in Soils Workshop sponsored by SETAC and administered by the SETAC Foundation for Environmental Education held on 19–23 June 1999. Hazard assessment applications include classification for the purpose of labeling of materials in commerce, soil criteria derivation, ecological risk assessment, and site remediation. The chemistry of metals differs from that of organic compounds, necessitating different approaches for hazard assessment. Equilibration of metal-containing substances in soils during test setup is a major difference. A transformation protocol was proposed to simulate weathering of metal compounds in the environment in order to ascertain whether the bioavailability of these naturally persistent compounds might change over time and to estimate salt effects. Consensus on soil type was not achieved. Use of artificial versus natural soil, as well as the number and types of soils required, still remains unresolved for hazard classification. There was general agreement, however, that ecological risk assessment requires use of natural soils representative of the area under consideration because our ability to extrapolate toxicity data across soil types is limited. The Plant and Soil Invertebrate Workgroups proposed standard species and described detailed test guidelines. Several microbial function tests were recommended, but additional information will be required to standardize the methods and put the results into an ecological context. Determination of the potential for metals to biomagnify in the food chain is complex because organisms have evolved various mechanisms to use, exclude, or take up these naturally occurring substances. Workshop participants strongly endorsed the concept that measurement endpoints chosen for all tests be ecologically relevant for acute and chronic effects.

Keywords: hazard; metals; soil; toxicity tests; risk assessment

Materials used in manufacturing and commerce may be hazardous to the environment. Hazard is a measure of the inherent capacity of a substance to cause an adverse response in a living organism (OECD 1995a). Organisms can be placed at risk if the substance enters the environment, with the degree of risk being related to the hazardous nature of the substance and the amount of exposure that occurs. Therefore, substances that are very hazardous have a greater likelihood of causing environmental injury in the case of spills or other accidents than those that are less hazardous.

While this logic is easy to follow, it is less obvious how hazard is defined. Hazard is the intrinsic relationship between a chemical and an organism and therefore differs, depending upon both the chemical and the organism. In order for a chemical to affect an organism, it must be in contact with the organism and then reach the particular cell or organ where it can bind to receptors and cause a response. Through this process, the chemical may be transported by a particular protein in the organism, it may be sequestered in areas where it can do no harm (such as cell walls or fat bodies), or it may pass through an organ such as the liver where it is broken down and detoxified (or, in the case of some organic substances, made more toxic). Different durations and amounts of exposure result in different effects, from immediate mortality or the altering of growth, reproduction, and development, to chronically reduced capabilities of carrying out normal life functions.

Metals have some special properties that make it particularly difficult to quantify their level of hazard to soil-dwelling organisms such as plants and invertebrates or to the soil microbial community. Metals are naturally occurring, environmentally persistent substances; therefore, organisms have developed various adaptive mechanisms for dealing with their presence in the soil. Some organisms actively exclude many of the metals from being taken up. Others concentrate metals and sequester them in places that cause no effects to the organisms but may make them less palatable to animals that consume them. Some of the metals (e.g., copper, cobalt, nickel, zinc) are required micronutrients for plants or animals. For these substances, organisms have developed homeostatic mechanisms to keep their body concentrations in the optimal range. If soil concentrations are low, these metals are actively taken up; at high concentrations, uptake mechanisms may be blocked or the metals may be excreted more rapidly. Finally, some metals (e.g., lead) have no known biological functions and, if present in sufficiently large concentrations and bioavailable, are toxic to organisms.

Furthermore, specific physical and chemical properties of metals make them difficult to work with when following standardized toxicity test protocols. Incorporation of sparingly soluble metal compounds into the soil is difficult, and aging of the material significantly affects uptake and toxicity. In addition, soil properties that affect the availability requirements for and interactive effects of certain essential metals also have the potential to confound test results.

Nevertheless, information about the hazard of metals and metal compounds to soil organisms is necessary for labeling and handling of materials in commerce, for assessing risks to terrestrial systems from new or existing sources of metals, and for cleanup of contaminated sites. This summary outlines the results and conclusions of a technical workshop held in Barcelona, Spain, on 19–23 June 1999 that critically examined the current standard soil toxicity test methods. A detailed workshop report is being prepared for publication by the Society of Environmental Toxicology and Chemistry (SETAC). Modifications to standard guidelines were proposed in order to accommodate the particular biogeochemical properties of metals and their derivative substances, including a discussion of 1) the types of soils for which the data will be applicable, 2) appropriate test species and their source and acclimation conditions, 3) methods for incorporation of material into soil, and 4) awareness of micronutrient requirements and interactions. The use of toxicity data in hazard identification, risk assessment, and criteria setting also was discussed in order to delineate clearly the context within which test guidelines may be applied.

Workshop Purpose and Goals

Many efforts on the national and international scales are underway to develop soil quality criteria or to conduct either site-specific or regionally based risk assessments for metals and their derivative substances in terrestrial environments. A major requirement of any of these efforts is an accurate measurement of hazard. Additionally, the European Union (EU) is moving forward with development of a classification system to label materials in commerce and transport with respect to their potential hazard to the terrestrial environment. Although there are numerous studies in the literature on toxicity of metals or metal products to plants, animals, and soil microbes, most of the data are inappropriate for comparative hazard ranking or for use in criteria development or risk assessment. Most of the data were generated by using nonstandard tests (e.g., the soil matrix differed, a wide range of mostly agronomic species were used, and a variety of endpoints were measured). This resulted in a dataset that is difficult to use for regulatory purposes because specification of hazard thresholds is impossible.

To date, there are only a few standard international guidelines for soil studies, although many others have been proposed (Léon and Van Gestel 1994). The Organization for Economic Cooperation and Development (OECD) has published 2 standardized tests: the earthworm acute toxicity test in artificial soil (OECD 1984a) and the early seedling emergence test, also in artificial soil (OECD 1984b). The American Society for Testing and Materials (ASTM) has standards for whole-plant toxicity tests in soils (ASTM 1994, 1997, 1999). Test methods for other soil invertebrates (e.g., nematodes) and for soil microbial processes have been proposed but have not received any formal review by OECD, the EU, or ASTM. Furthermore, none of the accepted or proposed test protocols address particular problems of testing metals and their derivatives or other sparingly soluble substances. For example, soil type (including such factors as organic matter, pH, and redox potential) and the length of time the material is in the soil profoundly affect bioavailability and, therefore, resultant toxicity measurements.

Data from toxicity studies are likely to be used 1) in comparative hazard ranking, 2) for establishing cleanup goals, and 3) for risk assessment. Although all 3 of these management processes use hazard information, their applications are sufficiently diverse that it is possible that toxicity tests would be designed differently for each (Smith and Hart 1994). Hazard identification and ranking should be based on the intrinsic properties of the substance. Identification of the real ability or likelihood that a substance could cause detrimental effects to the ecosystem is the objective of a risk assessment or criteria-setting process and must account for additional factors such as production rates, environmental emissions, degradation and transformation rates, exposure pathways, different environmental conditions, bioavailability, etc. (van Tilborg and Van Assche 1995). Therefore, toxicity tests used for comparative hazard identification may be optimized by using standard soils, while hazard information developed for risk assessment or criteria setting may use varied test parameters, depending upon soil type, or may require some method for adjusting a single measured toxicity value by appropriate soil parameters (e.g., pH and organic carbon) (Scott-Fordsmand et al. 1996).

The questions addressed in this workshop then become 3-fold. First, how should existing standardized soil toxicity tests be modified to accommodate the particular properties of metals and inorganic substances (e.g., required micronutrients, incorporation into soils of sparingly soluble substances, species adaptations and acclimatization)? Second, what additional standardized tests are required to characterize hazard to important biotic components of the soil ecosystem? Third, how should the hazard assessment information be integrated with exposure information (i.e., environmental chemistry, bioavailability, interaction with other naturally occurring metals or metalloids, etc.) to provide an assessment of risk to terrestrial environments, to develop universal soil criteria, or to develop comparative hazard identification?

Participation and Format

The workshop brought together 31 experts from 11 countries for 4 days of discussion on methods for assessing hazard of metals and metal compounds to soil organisms. Participants included regulators, academics, industry representatives, and consultants who face issues of how to develop or apply terrestrial hazard assessment concepts in the regular course of their profession (see Participants List, p 21). The uses and limitations of this type of hazard information in classification, risk assessment, and contaminated-site assessment were discussed as well. The workshop consisted of the following 5 workgroups:

- Applications of Hazard Assessment Information Workgroup,
- Soil Chemistry Workgroup,
- Soil Microbial Tests Workgroup,
- Soil Invertebrate Tests Workgroup, and
- Terrestrial Plant Tests Workgroup.

Applications of Hazard Assessment Information Workgroup

Determination of the hazard of metals and metal compounds in the terrestrial environment should include a balanced input of data from all compartments, including soil properties and toxicity information from plants, invertebrates, and the microbial community. There are 4 principal uses of this information:

- environmental hazard classification of substances,
- contaminated-site assessment and effectiveness of remediation efforts,
- soil quality criteria derivation for individual substances, and
- ecological risk assessment, both site-specific (i.e., local) and generic (i.e., regional).

The objective of hazard classification is to rank the potential hazard by using standard test conditions with clearly defined endpoints. A key outstanding question is whether or not a classification system should use hazard data obtained from tests that maximize response, recognizing that this requires different test conditions for different metallic substances, or whether 1 set of standardized conditions should be used for all substances. Contaminated-site assessment and remediation efforts require information obtained through direct testing of contaminated soil under conditions consistent with standard test methods.

Derivation of soil quality criteria is comparable to development of the probable no-effect concentration (PNEC) for a generic risk assessment. However, derivation of a single PNEC for each metal does not make ecological sense because of the large variability in response of different organisms to metals in different types of soil. This variability is a result of factors influencing the external bioavailability, biological membrane processes, and internal homeostasis mechanisms of soil organisms. These topics formed the basis of a previous SETAC-sponsored workshop on contaminated soils (Saterbak et al., in prep.). Thus, laboratory ecotoxicity results must be considered initial approximations until site-specific adjustment algorithms can be developed. Moreover, the workgroup recognized the desirability of developing PNEC values and soil quality criteria from toxicity tests of effects on populations or community structure, rather than individual organisms, in order to provide more ecologically relevant values.

The workgroup recognized that organisms have developed, over time, physiological adaptive mechanisms to maintain internal body concentrations of essential elements at optimal levels. Similar mechanisms have been developed for nonessential elements to limit their uptake, enhance their excretion, or sequester them in inactive tissues. A metal becomes toxic for a given organism if the concentration at the target structure exceeds the organism's homeostatic capacities. Thus, organisms should be adapted to the test system in order to reduce the introduction of additional stress factors into the toxicity test results. Measurement of relevant stress enzymes or changes in energy patterns may serve as early biomarkers to determine if the culture or test environment is stressful to the organism (Van Gronsveld et al. 1995, 1996).

Although the focus of this workshop was on developing methods to measure effects to organisms from direct soil exposure, it is acknowledged that the overall objective is to evaluate hazard for the terrestrial ecosystem. Therefore, there is a need to identify and suggest further tests (e.g., foliar invertebrates) and to choose test endpoints that are ecologically relevant for both acute and chronic effects. However, information about potential hazards associated with subsurface soils and ground waters should be addressed only for risk assessment cases. Soil porewater hazards should be estimated when the organism of concern receives its entire exposure by this route alone (e.g., for some soil nematodes).

One important aspect of organic compounds that significantly contributes to the assessment of the potential ecological risk is the length of time the material persists in the environment and its capacity to accumulate and concentrate as it moves through the food chain. However, it is difficult to translate these concepts directly to the process for ascribing risk from metals. Metals are persistent by nature, so it is the capacity to release the bioavailable fraction of the metal that is important from a ecotoxicological point of view, not the persistence per se. Similarly, many of these substances bioaccumulate in the food chain, particularly at the lower trophic levels. However, bioaccumulation of metals is a poor predictor of toxicity because of the homeostatic mechanisms available to regulate the amount of required micronutrients and to cope with the natural occurrence of nonessential metals. Moreover, bioaccumulation processes are not constant; rather, they depend upon the relative amounts of the metal in the soil and organism. For this reason, it is difficult to predict uptake rates and potential accumulation into the food chain across all possible soil concentrations. Measurement of tissue concentrations in test organisms is encouraged as a means of developing information about critical body burdens (i.e., the tissue concentrations at which toxicity results are manifested) and bioaccumulation potential.

For metals, it is recognized that a background concentration in the soil and food of cultured animals is both required (for essential metals) and unavoidable (for essential and several nonessential metals). The highest (for all metals) and lowest (for essential metals) concentrations acceptable in both the standard soil and the culture medium or food of the organisms must be established. Minimum concentrations are required such that the test organisms are not stressed nutritionally prior to the introduction of the test substance.

Hazard information for classification purposes generally is developed by testing standard species. This may limit the ecological relevance of this information. For risk assessment, high-quality data on many species are desirable. Site-specific assessments should test organisms that are relevant for the area under consideration.

The Applications Workgroup supports the suggestion by the Soil Chemistry Workgroup to conduct toxicity tests at 2 times after addition of the test substance to the soil. Tests should be conducted after a short equilibration period of the test substance in the soil (e.g., 2 days) by using relevant short-term protocols and endpoints (Time 1). Additionally, 1 set of soils would be allowed to equilibrate with the test substance for an additional extended period (e.g., 60 days) prior to testing (Time 2). Yet another set of soils would be leached with deionized water and subjected to the additional extended equilibration time. At this point (Time 2), the toxicity tests would be conducted again with both the leached and unleached soils, this time with the focus on relevant long-term endpoints but also including measurements of the same short-term endpoints that were conducted at the initial test period. Thus, Time 2 results would be used to verify whether the original classification (based on Time 1 results) is appropriate, or whether bioavailability increases with time. Time 1 results would be applicable to classification criteria requiring acute or short-term hazard data, while Time 2 results would contribute to assessment of longer-term hazards. The parent compound should be used in all tests rather than always testing a soluble form of the metal, as is the case with aquatic organism testing. The workgroup recognized the need for further directed research to refine the methods used in the transformation/leaching process. However, they strongly endorsed this concept as a means for providing relevant information about toxicity of metals and sparingly soluble metal compounds to soil organisms, while accounting for the persistence and bioavailability changes that are inherent properties of these substances.

Soil Chemistry Workgroup

Most metals are persistent in soils (McGrath 1987), although significant leaching losses are possible (Holm et al. 1998). In addition, changes in chemical form can occur over time (Barrow 1998; Hamon et al. 1998) and these changes can profoundly influence the solubility and bioavailablity and hence the toxicity of the metal. First, there may be an assortment of dissolved forms of the metal in the soil solution that differ with respect to their bioavailability (Lorenz et al. 1997; Smolders et al. 1998). Second, most metals are predominantly associated with the soil solid-phase (Anderson and Christensen 1988). With some soil fauna, trace-element exposure involves ingestion of the soil solid-phase so that the effective dose is a complex function of the solid-phase forms and the chemistry of the gut. There is little to suggest that currently available chemical-extraction schemes can predict bioavailablity to a wide range of species accurately. Third, some of the metals commonly found in the environment have more than 1 oxidation state. Chemical characteristics can vary markedly among these different forms so that the toxicity varies substantially and results based on 1 oxidative state or ionic species cannot be directly transferred to another.

Soils are heterogeneous mixtures composed of a large variety of organic, organomineral, and mineral components as well as soluble substances; therefore, the quality and quantity of metal binding is highly variable among and within soils. For this reason, in hazard identification and classification, but not in risk assessment, use of a common soil in all toxicity tests would greatly enhance the comparability of the results. Artificial soils have many advantages, including preexisting acceptance in ecotoxicological testing, while disadvantages include use of kaolinitic clay that is not typical of temperate zones, unsuitability for endemic microorganisms, and problems maintaining the viability of introduced soil invertebrates. Natural soils would be suitable for all organisms, although there are significant difficulties in providing a large, homogenous supply of soil to all interested users. The Soil Chemistry Workgroup suggested that, rather than identifying a particular location for soil collection, a range of chemical, physical, and toxicological characteristics would be specified; a simple cation/anion availability calibration test would be performed; and each laboratory could locate and collect soils for its own use, provided that the soils meet the required specifications. To determine hazard of substances at their highest practical bioavailability, cationic metals such as copper, cadmium, lead, nickel, and zinc would require use of a soil with low pH (e.g., 5.0 to 5.5), low organic matter content (e.g., 2%), low cation exchange capacity, and low amounts of iron, manganese, and aluminum oxides. Anionic metalloids such as arsenic and selenium would require soils with high pH (e.g., 7.50 to 8.0), although all other parameters would be similar. Soils that contain naturally high concentrations of metals should be avoided, and soils should not have had any recent application of pesticides or other toxic compounds. Toxicity response to addition of a reference substance (such as a soluble metallic salt) should fall within predetermined limits for the regional soil to be acceptable as a standard test medium.

When setting up a toxicity test, metal compounds should be mixed with soil that has been prepared to a specific grain size. Colloidal silica may be used as an inert carrier. A period of 2 to 7 days should be allowed after the mixing process before introduction of the test organisms or, in the case of indigenous soil microorganisms, before the testing process begins.

However, a 2- to 7-day equilibrium period for the test substance may underestimate toxicity. Substance dissolution in soil may be a slow process, requiring long equilibration times. Therefore, the Soil Chemistry Workgroup suggested allowing the test substance to equilibrate in the soil for 60 days after the initial 2- to 7-day incubation and then rerunning the toxicity tests. Moreover, 1 batch of soil should be leached with 3 porewater volumes of deionized water at the start of the 60-day transformation period. The aim of the leaching test is 1) to separate the effects of the metal from any possible toxicity-enhancing or neutralizing effects of other readily soluble or degradable components in the test substance and 2) to enhance substance "weathering." If toxicity increases with time, the substance would be considered more hazardous. If, however, the toxicity decreases with time, no change would be made to the hazard classification, because the proposed equilibration time is based neither upon specific knowledge of when the initial rapid reaction of the substance with the soil has been completed nor on knowledge of when valence-state stability has been achieved.

This proposed testing and transformation protocol should not be used for setting soil standards, because the fate of the compound is tested in a limited number of soil types and after only 2 equilibration times. Furthermore, extrapolation to risk assessments of metals in the environment is tenuous because the long-term fate (i.e., years) and subsequent changes in toxicity of the test substances would not have been demonstrated in these tests.

Soil Microbial Tests Workgroup

Soil microbes are defined as soil fungi, bacteria, actinomycetes, and protozoa. Microorganisms can amount to 90% of the living matter in soil (plants excluded). They contribute to essential soil functions such as decomposition of organic residues, nutrient cycling, and soil aggregation (Torstensson et al. 1998), as well as being important constituents of food chains. Many microbial tests exist, but standard procedures are only in draft form. A diverse literature exists, but it is not aimed at hazard assessment of metal-containing substances in the soil environment.

There are no methods for quantitatively extracting microbial communities from soil, nor is it possible to add a standard and ecologically relevant microbial culture to test soils. Therefore, it is recommended that tests be conducted with natural soils that contain their indigenous microbial communities. This has the added benefit of mimicking the natural system, including the natural diversity that is present. The recommended tests build either on available draft standardized protocols or on those protocols that could be standardized in the near future. New or nonstandard tests were reviewed and recommendations made as to their importance for hazard assessment (Table 1).

Table 1 Recommended soil microorganism ecotoxicological bioassays

Test	Reliability	Ease	Relevance	Sensitivity
Carbon-driven process assessment				
Substrate-induced respiration	High	High	High	Medium
Basal respiration	High	High	High	Low
Nitrogen-driven process assessment				
Mineralization of organic nitrogen	High	High	High	Low
Ex-situ assays				
lux-based system	High	High	High	High
Microtox®	High	High	Medium	High

Carbon mineralization is an important function of soil microbes and has been well-studied in relation to metal-polluted soils (Scott-Fordsmand and Pedersen 1995; van Beelen and Doelman 1996; Scott-Fordsmand 1997). This test also is referred to as "soil respiration." It is studied by measuring the amount of carbon dioxide produced over a specific time period and is a relatively easy and reproducible test. The test may be conducted with indigenous respiration rates or by introducing either a single carbon source or a range of carbon sources that allows more detailed characterization of the soil response (Anderson and Domsch 1978). The draft OECD respiration technique (OECD 1999) could be a basis for a metal hazard test but requires more study to standardize the equilibration period of the metal in the soil and to refine the methods for addition of the carbon source.

Total microbial biomass has been measured in metal-impacted soils (Brookes and McGrath 1984). The biomass-carbon component of the soil may change if the metals causes a reduction in numbers of organisms or if metal-resistant microbial populations expand in size. The fumigation method provides a reliable estimate of soil biomass.

Nitrogen cycling and transformations of sulfur and phosphorus are very important functions of soil microbial communities. Quantification of components of the nitrogen cycle requires great care. Certain portions of the nitrogen cycle are based on extraction and measurement of nitrate from the soil matrix. Sufficient oxygen and a suitable moisture and temperature regime must be maintained. This is a well-defined technique, and many studies have shown that nitrification is inhibited in soils contaminated with metals (Giashuddin and Cornfield 1978, 1979; Chang and Broadbent 1982). There are no standardized methods available for quantification of sulfur and phosphorus transformation processes.

Microorganisms release enzymes into soil to catalyze degradative processes and facilitate intake of required substances. Inhibition of these processes, resulting in a lower measurable amount of enzymes in the soil, may indicate toxicity from metals (Juma and Tabatabai 1977; Scott-Fordsmand and Pedersen 1995). The measurement of enzymes is relatively inexpensive, rapid, and highly reproducible. However, the ecological relevance of the results often is difficult to interpret. Activity of these enzymes at any single time cannot be related directly to the fluxes of nutrients or toxicants through soil. Additionally, plant roots also contribute many of the same enzymes to the soil.

The measurement of adenosine triphosphate (ATP), a product of cellular metabolism, is an ideal assay for differentiating between living and dead cells. Reasonably good correlations have been found between soil ATP concentrations and biomass carbon. The method potentially could be used to assess ATP content of actively growing cells as a measure of metal toxicity. There is a wide range of commercial kits available that make this assay convenient and simple to carry out.

Bacterial and fungal abundance (measures of community structure) have been estimated by using conventional microbiological methods from plate counts. However, this method is problematic because not all spores or fungal hyphae will form colonies on plates and only a fraction of the total soil bacteria will grow on any of the generally accepted nutrient agars. The selection of a specific method for extracting the microbes from the soil matrix also influences the community available for measurement. Furthermore, pollutants may change the component of the microbial community that is viable but nonculturable; hence, the pollutant-induced change would not be detected by these conventional techniques.

Recently, more technically advanced methods have been applied to the measurement of diversity of microbial communities in soils. Molecular techniques are being used to study changes in the nonculturable component of the soil community. Methods include measurement of the fatty acid profile of the soil organisms, phospholipid ester-linked fatty acids, and DNA extraction. Various abiotic soil factors affect the success and reproducibility of the process. These techniques are highly specialized and are regarded at present as research tools rather than as easily applicable diversity indices. Various abiotic soil factors determine the success and reproducibility of the process, particularly the extraction of genetic material from the soils.

It is also possible to test soil microbial isolates (e.g., protozoa, fungi, and bacteria) in pure culture . Commercially available test kits use bioluminescent bacteria. The principle is that a pollutant may inhibit metabolic activity and, as a consequence, decrease bioluminescence output. The insertion of the *lux* genes into a range of soil bacterial isolates allows many bacteria to express bioluminescence and has made these measurements more ecologically relevant to soil systems across a broad pH range.

The workgroup favored the inclusion of a biosensing technique that could act as a rapid and sensitive indicator of potential metal impacts. The pore water is extracted and a soil organism such as *Pseudomonas fluorescens* is selected and fitted with the *lux* gene. Bioluminescence can then be used as an indicator of toxicity. However, this method is still in its developmental stage; in the meantime, the workgroup recommended using the Microtox® test (or an equivalent) on soil aqueous extracts as a screening tool for relative hazard.

After reviewing the range of techniques available for studying soil microbial ecotoxicology, the workgroup selected specific tests for assessing hazard of metals in soils. The selection focused on the functional role of microbes for several reasons. First, the methods are relevant to all soils, irrespective of their geographical location and pedoecoregion. Second, they have well-defined endpoints, require little specialized equipment, and are cost-effective. Third, they are perceived as being highly reproducible. On the other hand, their relative sensitivity to metals is not known. The selected techniques are

- respiration, both basal and substrate induced,
- nitrogen mineralization of plant residue that has a low C:N ratio, and
- biosensor detection of toxicity.

Each of these tests has its limitations and would benefit from some short-term, directed research prior to drafting standardized protocols. A further area of research should consider some of the other assays covered in this review. The selection of the methods was not an easy task, and exclusion should not be considered a reflection of the quality of the data that can be derived from these methods.

Soil Invertebrate Tests Workgroup

The soil-litter subsystem is one of the most complex and taxonomically diverse components of the soil ecosystem and has a large influence on trophic relationships. Soil invertebrates occupy a central role in the energy pathways in such systems as they feed on all types of soil organisms and material (plant, bacterial and fungal feeders, predators, and omnivores). They may be exposed to soil-associated contaminants through dermal uptake from the soil pore water and/or through ingestion and oral uptake of soil particles. Laboratory toxicity tests with soil organisms form the basis for the hazard and risk assessment of metals in soil ecosystems (EU 1996), although the earthworm is the only soil invertebrate test formally accepted by the OECD (OECD 1984a). The need for enlarging the battery of tests—using soil invertebrates that represent a wide range of life styles—has been recommended by various authors (Van Straalen and Løkke 1997; Løkke and Van Gestel 1998). Although short-term survival tests may provide valuable initial data on the ecotoxicity of soil contaminants, successful protection of populations requires information on the reproduction of the species (Van Straalen et al. 1989).

A key question in soil invertebrate toxicity testing is the type of soil to use in standardized tests. For hazard identification, the same soils should be used for all organism tests, with the number of different soils kept as low as possible. Realistic worst-case situations should be represented, but background concentrations of metals must remain a consideration. Artificial soils are widely accepted in soil invertebrate testing and are relatively easy to prepare. However, not all of the important test organisms survive or reproduce in artificial soil. Natural soils provide a more realistic exposure situation, are more useful for purposes of environmental risk assessment, and are in accordance with discussions in the area of fate testing. However, guidance is needed about which soils to use. The workgroup suggested consideration of the 6 EUROSOILS, a group of well-characterized soils, according to an OECD workshop held in Belgirate, Italy, in 1995 (OECD 1995b). Soils with similar physicochemical characteristics may be found in North America and Japan. A second possibility is to adopt the approach suggested by the Soil Chemistry Workgroup and define 2 natural soil types with characteristics that would provide reasonable maximum bioavailability for cationic and anionic metals while still being suitable for life-support of most soil organisms. These would roughly correspond to EUROSOIL 3 (for cations) and EUROSOIL 2 or 4 (for anions). The properties of soils that determine bioavailability are not completely understood. It is not yet possible to extrapolate bioavailability from one soil to another by means of simple algorithms.

Incorporation of metals and sparingly water-soluble metal compounds into test soils is one of the most crucial aspects of test standardization. Metal salts, organometallics, and metallics should not be treated similarly. However, if only 1 form of a metal is to be tested, a soluble salt is preferred. Most test results described in the scientific literature have been derived from test soils spiked with readily soluble metal salts. This implies that observed biological responses were a function of both the metal and the anionic moiety that was added with the metal. The "salt effect" introduced in this way should not be ignored, and various researchers have proposed methods for taking this into account (Van Straalen et al. 1989; Smit and Van Gestel 1998). However, these methods are not applicable when sparingly soluble metal compounds are being tested. The workgroup discussed several approaches that could be taken to address the spiking problem, recognizing that further research in this area is essential. Regardless of the approach used, care has to be taken not to destroy the structure of the soil to the point where it becomes unsuitable as a habitat for invertebrates. The transformation protocol proposed by the Soil Chemistry Workgroup is considered acceptable, although it is recommended that "engineering" invertebrates (e.g., earthworms and other organisms that change the soil structure) be included in the soil during the transformation period. It may be that these invertebrates accelerate the transformation process by increased mixing or gut passage.

The workgroup recommended 4 test species for hazard assessment that are representative of various taxonomic groups, ecological functions, trophic levels, and exposure routes (Table 2).

Table 2 Recommended test species of invertebrates

Animal	Protocol
Eisenia anrei (Lumbricidae; earthworm)	ISO (1996)
Enchytraeus albidus (Enchytraeidae; potworm)	OECD Draft (Römbke and Moser 1999)
Folsomia candida (Collembola; springtail)	ISO (1999)
Hypoaspis aculeifer (Gamasina; predatory mite)	Løkke and Van Gestel (1998)

The workgroup provided specific suggestions for conducting the toxicity tests, with detailed guidelines to be provided in the chapter annex to appear in the Workshop Technical Report. A range-finding test is recommended to determine the concentrations to be used in the definitive test. If no effects are observed up to 1000 or 10,000 mg/kg, the definitive test should be designed as a limit test. It is recommended that the definitive test be set up as a regression analysis to determine an effects concentration rather than a no-effect level. In other words, the hypothesis we are testing is not whether the chemical is toxic but at what concentration it becomes toxic and how much of an increase is required for this response to be expressed. The test endpoint recommended is the number of juveniles produced by the end of the experimental period. The time of testing will vary from 21 days (*Folsomia candida*, *Hypoaspis aculeifer*) to 8 weeks (*Eisenia andrei*). The concentration at which 50% of the test replicates are affected is recommended as the appropriate measure for hazard identification.

It is recognized that increased metal tolerance by invertebrates can occur either through physiological acclimation or genetic adaptation (Reinecke et al. 1999). It is suggested that a screening procedure be established to ensure that acclimation/adaptation does not occur in the culture systems. Research is required to establish the physiological ranges for essential metals in soil organisms that do not induce deficiency. Because of the long-term nature of the tests, food will need to be added to the system after being analyzed for metals and determining that contaminant residues are low. Research is still needed to clearly identify the range of environmental parameters required for the normal growth and reproduction of some of the suggested test species.

The testing strategy described must be considered the first step in a tiered approach. Results can be used in hazard identification or, in conjunction with exposure assessments, as an initial characterization of risk. However, a complete assessment of risks to biodiversity (Glowka et al. 1994) is outside the scope of the present document. There is a need for functional testing for invertebrates, which participate in decomposition processes along with the microorganisms, but such tests also fall outside the scope of hazard identification. Furthermore, bioaccumulation is considered a suitable approach for assessment of bioavailability in the field and transfer of metals through the food chain; however, it too falls outside the scope of hazard identification. Lethal body concentrations are known for only a few invertebrate species and chemicals (Van Straalen 1996).

Terrestrial Plant Tests Workgroup

Key issues in terrestrial plant toxicity testing were identified and discussed, taking into account existing standard test guidelines (OECD 1999; ASTM 1999). Three applications were considered: hazard assessment, regional and site-specific ecological risk assessments, and site remediation, with most of the discussion focused on hazard assessment.

Chronic exposures for plants are those that span a majority of the life expectancy of the species or a significant portion of the vegetative or reproductive life stages. Endpoints related to growth and biomass production can be considered in both acute and chronic exposure scenarios. Furthermore, increasing the test duration from 14 to 21 days increases the resolution of the tests because there is a better expression of effects. On the other hand, an extended exposure may require nutrient amendments or greater soil volume in each test unit. Therefore, a 14-day exposure period was recommended by the workgroup for the purposes of hazard classification, and a longer exposure period was suggested for risk assessment. Contaminated-site assessments and phytoremediation may require different species and a determination of tolerance rather than sensitivity, but the basic testing procedures are the same (Table 3).

Table 3 Use and requirements for hazard assessment in different regulatory processes

	Classification for soil effects (EU-OECD)	Ecological risk assessment (EU-OECD)	Contaminated sites evaluation/ remediation
Required test	Standard	Standard + non-standard	Standard + non-standard
Required result	1 (lowest) toxicity value	PNEC derived from whole database	Toxicity screening
Reference environment	1 generic (environmentally relevant)	Local or Regional	Local

Standard test guidelines designed for assessing the toxicity of pesticides and industrial chemicals to nontarget plant species currently are available. These tests include an early seedling growth test (ASTM 1994), a seedling emergence, root elongation test (OECD 1984; USEPA 1986; USFDA 1987a, 1987b; APHA 1992; OECD 1999; ASTM 1999), and a life-cycle test (ASTM 1999). These existing standards can be used to determine phytotoxicity for soluble metals and sparingly soluble metallic substances for hazard classification. Most of the standard methods are acute tests focused on the early stages of plant development. Only 1 full life-cycle test, the Brassica life-cycle test (ASTM 1999), has been standardized, although several modifications with other species are ready for standardization. If a seedling emergence test is used, nondestructive shoot height, development measurements, and survival during the early periods (e.g., weekly observations from planting time) can be used as measurement endpoints for both acute and chronic exposure. The workgroup considered a strict distinction between acute and chronic exposure less relevant to plant testing because short-term plant tests that include vegetative growth parameters provide information relevant to the plant's whole life cycle. However, the desire of regulatory groups to have both acute and chronic exposure-response data, and their tendency to want reproduction endpoints, should be recognized. The workgroup supported the use of the currently available standardized Brassica life-cycle test to generate both acute (e.g., 14 to 21 days) and chronic (e.g., > 21 days) exposure response data for vegetative and reproductive endpoints, respectively.

The available standard plant tests allow maximum flexibility in study designs, a significant disadvantage for the purposes of generic hazard assessments. The acceptable range of pH, organic matter, and clay content would permit testing under markedly different bioavailability conditions. Recognizing the need to minimize variability of test results for hazard classification, the workgroup recommended an artificial soil matrix consisting of 75% silica sand, 20% kaolinite, and 5% organic matter (e.g., peat or O-horizon). Use of this artificial soil for all hazard determinations provides the best opportunity for relative comparisons of hazard for all test substances, particularly metals whose bioavailability is markedly influenced by these soil parameters. Moreover, the use of this artificial soil provides a means of referencing test results for tests conducted on complex soils, including those from contaminated sites. The artificial soil originally proposed by the OECD for use in toxicity tests with earthworms (OECD 1984a), provides the basis for a standardized test matrix for use in acute (e.g., 14-day) toxicity tests with plants. Although there is increasing support in the scientific community and in the other workgroups from this workshop to use "natural" soils for hazard assessment, there are limited data from such soils, and no agreement has been reached on how to compare results among different soils or with those achieved from artificial soils. Until this comparative study is conducted, the Plant Workgroup believes that the merits of using a highly standardized test matrix outweigh the perceived advantage of a natural soil.

However, for hazard determination for regional or site-specific risk assessments, chronic exposure tests should be performed on natural soils representative of the region or site. Test conditions that incorporate bioavailability factors into the study design provide empirical data that reduce uncertainty that otherwise remains unaccounted for in the exposure portion of the risk equation. For contaminated-site assessments, phytotoxicity tests can be performed on the site soils by using the preexisting concentrations of metals or with site soils where the constituent is added.

The test soil should be prepared and allowed to equilibrate for 5 days prior to introduction of the test substance; seeds are planted 2 days later. Preliminary recommendations of test species by the Plant Workgroup include alfalfa (*Medicago sativa*), barley (*Hordeum vulgare*), radish (*Raphanus sativus*), and northern wheatgrass (*Agropyron dasystachyum*) or perennial ryegrass (*Lolium perenne*). The process for test species selection has been described in detail elsewhere (Stephenson et al. 1996). For regional or site-specific assessments, investigators may propose and justify a suite of species, including indigenous plants, that meet the specific objectives of the assessment. Testing of species found in areas of the highest and lowest metal concentrations present on the risk assessment site is recommended.

Hazard assessment tests should be conducted in growth chambers or greenhouses to standardize environmental conditions. However, tests conducted for site-specific assessments may require that air temperature and relative humidity be changed to simulate conditions at the site of interest.

Measurement endpoints include seedling emergence, survival, root and shoot length, root and shoot wet and dry mass, and total wet or dry mass (e.g., root plus shoot wet or dry mass). For the range-finding test, it is not necessary to measure all of these endpoints. However, at a minimum, seedling emergence, survival, and root and shoot length should be measured. For the definitive tests of either short (14-day) or long (21-day) duration, it is recommended that all of the endpoints be evaluated. A procedure to express multiple measurement endpoint responses as a single phytotoxic score may be a useful means of representing hazard assessment data for several species (Kapustka 1996).

In addition to these biological measurements, physicochemical measurements of pH, conductivity, and nutrients for soils in each treatment should be made at the beginning and end of a test. The changes in these parameters over the duration of the test can assist in the interpretation of anomalous results of the tests. Exposure concentrations should be measured for all metals at the beginning and end of the test to establish concentration-response relationships.

The range-finding test should consist of a minimum of 6 exposure concentrations, including the control treatment, with equal replication across treatments. There should be at least 3 replicate test units per treatment, although 4 to 6 replicates may be advisable because the power of the test would be increased. The definitive test should consist of between 10 and 13 treatments, including the appropriate control treatments, with 6 replicates in the control treatment and a specified lesser number of replicates in the various exposure groups. Statistical evaluation for the different measurement endpoints of the definitive test should be determined by using regression procedures to describe the concentration-response curve; use of hypothesis-testing methods to develop response/no-response comparisons should be used only in the range-finding study.

Although there is much information on the influence of soil factors on metal phytotoxicity, it is scattered and incomplete. Therefore, the execution of a systematic, multivariate study into the quantitative relationships between phytotoxicity and soil factors defining metal availability is strongly recommended. The goal should be establishment of an algorithm that can be used to normalize laboratory test data to the conditions of the real world.

Summary and Conclusions

Convening this workshop was very timely because potential hazards of metals and other substances to the soil ecosystem are coming under increasing scrutiny. During the past 5 years, Canada and many European countries have developed soil criteria for assessment and remediation of contaminated lands, and Australia and the U.S. Environmental Protection Agency are working towards this goal. These criteria depend upon information about hazards to plants, soil invertebrates, and soil microorganisms. Additionally, the EU is beginning the process of developing a hazard classification system for materials in commerce relative to their potential to cause adverse effects in the terrestrial ecosystem. Standard hazard identification protocols are needed for this effort if the goal of a comparative ranking is to be achieved.

The workshop participants recognized the need to standardize methods for hazard identification and testing across all substances, but they clearly identified specific properties of metals that require different approaches than those used for testing organic compounds. Mixing and equilibration of metals in soils during test setup is a major point of difference. The concept of a "transformation protocol" with short- and long-term tests run before and after the transformation period was cautiously endorsed by the workshop participants, with the caveat that additional work is needed to define the length of the transformation period for various substances, appropriate leaching and storage conditions, etc. The objective of the transformation protocol is to simulate weathering of metals in the environment to determine whether the bioavailability of these naturally persistent compounds might change over time. The use of the transformation protocol would be equivalent to evaluating environmental persistence of organic substances for the purposes of hazard classification.

Soil type also is a large consideration for testing of metals and was discussed at length by the workshop participants. Consensus and closure on this issue were not achieved. It was acknowledged that some widely occurring soils were not represented, in particular soils of a low organic matter status, such as those found in arid environments, or soils rich in iron and aluminum, such as those in the tropics. The use of a standard, artificial soil matrix was endorsed by the Plant Workgroup, particularly for the purpose of hazard identification and ranking. The Soil Invertebrate Workgroup preferred using natural soils that met specifications, such as those developed for EUROSOILs. The use of natural soils also was endorsed by the Soil Microorganism Workgroup because the proposed tests utilize indigenous organisms rather than the addition of cultures to a soil matrix. The Chemistry Workgroup also supported using defined natural soils, but pointed out the necessity of different soil parameters for maximizing bioavailability of different types of metals (cationic metals versus anionic metals). Thus, the use of artificial versus natural soil and the number and types of soils required remain unresolved for hazard identification and ranking. There was general agreement, however, that ecological risk assessment requires hazard information developed from natural soils representative of the area under consideration because our ability to extrapolate toxicity data across soil types is limited.

The Plant and Soil Invertebrate Workgroups proposed standard species and described detailed test guidelines (to be included in the Workshop Technical Report). The Soil Microorganism Workgroup recognized that there are no accepted standard methods for determining hazard to microbial communities. Several microbial function tests were recommended, but additional information will be required to standardize the methods and to put the results into an ecological context.

Workshop participants identified many areas in which short-term research will be required to formalize test methods. Moreover, although the focus of this workshop was on developing methods to measure effects to organisms from direct soil exposure, it was acknowledged that the overall objective is to evaluate hazard for the terrestrial ecosystem. Therefore, there is a need to identify and suggest further tests for soil ecosystem function, tests for above-ground organisms such as foliar and aerial invertebrates, and methods for identifying potential hazard to vertebrates from food-chain exposure or direct soil ingestion. Determination of the potential for metals to biomagnify in the food chain is much more complex than for synthetic organic compounds because organisms have evolved various mechanisms to use, exclude, or take up these naturally occurring substances. Finally, the workshop participants strongly endorsed the concept that measurement endpoints chosen for all tests should be ecologically relevant for both acute and chronic effects.

Acknowledgments — Financial support for the workshop is gratefully acknowledged from the following contributors (in alphabetical order): the International Copper Association, International Council on Metals and the Environment, the International Lead Zinc Research Organization, the International Zinc Association, the Nickel Industry Producers Environmental Research Association, Rio Tinto Ltd., Union Miniere, and the U.S. Environmental Protection Agency Superfund Program. The participants thank Dr. José Tarazona and his staff in Madrid, Spain, for help with local arrangements; Ria Vogelaerts at SETAC Europe for support in organizing the meeting; Greg Schiefer and Linda Longsworth for assistance in meeting planning; and Stacey Hagman, James Blackwell, and Rod Parrish for production and editing of the report.

References

Anderson PR, Christensen TH. 1988. Distribution coefficients of Cd, Co, Ni, and Zn in soils. *J Soil Sci* 39:1:15–22.

Anderson TH, Domsch KH. 1978. A physiological method for the quantitative measurement of microbial biomass in soils. *Soil Biol Biochem* 10:215–221.

[APHA] American Public Health Association. 1992. Aquatic plants in standard methods for the examination of water and wastewater. 18th edition. No. 8220. Washington DC: APHA.

[ASTM] American Society for Testing and Materials. 1999. Standard guide for conducting terrestrial plant toxicity tests. Annual Book of Standards. E1963-98. Conshohocken PA: ASTM.

[ASTM] American Society for Testing and Materials. 1994. Standard practice for conducting early seedling growth tests. Annual Book of Standards. E1598-94. Conshohocken PA: ASTM.

[ASTM] American Society for Testing and Materials. 1997. Standard guide for conducting laboratory soil toxicity or bioaccumulation tests with the Lumbricid Earthworm *Eisenia fetida.* Annual Book of Standards. E 1676-97. Conshohocken PA: ASTM.

Barrow NJ. 1998. Effects of time and temperature on the sorption of cadmium, zinc, cobalt, and nickel by a soil. *Aust J Soil Res* 36:941–950.

Brookes PC, McGrath SP. 1984. Effects of metal toxicity on the size of the soil microbial biomass. *J Soil Sci* 35:341–346.

Chang FH, Broadbent FE. 1982. Influence of trace metals on some soil nitrogen transformations. *J Environ Qual* 11:1–4.

[EU] European Union. 1996. Technical guidance documents in support of Directive 93/67/EEC on risk assessment of new notified substances and regulation. Risk Assessment of Existing Substances. EC No. 1488/94. Brussels, Belgium: EU.

Giashuddin M, Cornfield AH. 1978. Incubation study on effects of adding varying levels of nickel (as sulphate) on nitrogen and carbon mineralisation in soil. *Environ Pollut* 15:231–234.

Giashuddin M, Cornfield AH. 1979. Effect of adding nickel (as oxide) to soil on nitrogen and carbon mineralisation at different pH values. *Environ Pollut* 19:67–70.

Glowka L, Burhenne-Guilmin F, Synge H, McNeely JA, Gundling L. 1994. A guide to the convention of biological diversity. Cambridge, UK: International Union for the Conservation of Nature.

Hamon RE, McLaughlin MJ, Naidu R, Correll R. 1998. Long-term changes in cadmium bioavailability in soil. *Environ Sci Technol* 32:3699–3703.

Holm PE, Christensen TH, Lorenz SE, Hamon RE, Domingues HC, Sequeira EM, McGrath SP. 1998. Measured soil water concentrations of cadmium and zinc in plant pots and estimated leaching outflows from contaminated soils. *Water Air Soil Poll* 102:105–115.

Juma NG, Tabatabai MA. 1977. Effects of trace elements on phosphatase activity in soils. *Soil Sci Soc Am J* 41:343–346.

Kapustka LA. 1996. Plant ecotoxicology: The design and evaluation of plant performance in risk assessments and forensic ecology. In: La Point TW, Price FT, Little EE, editors. Environmental toxicology and risk assessment: Vol 4. STP 1262. Philadelphia PA: American Society for Testing and Materials (ASTM). p 110–121.

Léon CD, Van Gestel CAM. 1994. Discussion paper on the selection of a set of laboratory ecotoxicity tests for the effects assessment of chemicals in terrestrial ecosystems. Paris, France: Organization for Economic Cooperation and Development (OECD).

Løkke H, Van Gestel CAM, editors. 1998. Handbook of soil invertebrate toxicity testing. London, UK: Wiley. 281 p.

Lorenz SE, Hamon RE, Holm PE, Domingues HC, Sequiera EM, Christensen TH, McGrath SP. 1997. Cadmium and zinc in plants and soil solutions from contaminated soils. *Plant Soil* 189:21–31.

McGrath SP. 1987. Long-term studies of metal transfers following application of sewage sludge. In: Coughtrey PJ, Martin MH, Unsworth MH, editors. Pollutant transport and fate in ecosystems. Oxford, UK: Blackwell. p 301–317.

[OECD] Organization for Economic Cooperation and Development. 1984a. Guideline for testing of chemicals: earthworm acute toxicity test. No. 207 Paris, France: OECD.

[OECD] Organization for Economic Cooperation and Development. 1984b. Guideline for testing of chemicals: terrestrial plants, growth test. No. 208. Paris, France: OECD.

[OECD] Organization for Economic Cooperation and Development. 1995a. Test methods for hazard and risk determination of metals and inorganic metal compounds. Paris, France: OECD.

[OECD] Organization for Economic Cooperation and Development. 1995b. Final Report of the Belgirate, Italy workshop on selection of soils/sediments. Paris, France: OECD.

[OECD] Organization for Economic Cooperation and Development. 1999. Proposal for new guideline for the testing of chemicals: Soil micro-organisms, carbon transformation test. No. 217. Paris, France: OECD.

Reinecke SA, Prinsloo MW, Reinecke AJ. 1999. Resistance of *Eisenia fetida* (Oligochaeta) to cadmium after long-term exposure. *Ecotoxicol Environ Safety* 42:75–80.

Römbke J, Moser T. 1999. Organization and performance of an international ringtest for the validation of the enchytraeid reproduction test. Berlin, Germany: German Federal Environmental Agency. 150 p.

Saterbak A, Lanno RP, Fairbrother A, Gaudet C, Loehr R, Tabak H, editors. (In prep). Assessing contaminated soils. Pensacola, FL: Society of Environmental Toxicology and Chemistry (SETAC).

Scott-Fordsmand J. 1997. Toxicity of nickel to soil organisms in Denmark. *Rev Environ Contam Toxicol* 148:1–34.

Scott-Fordsmand JJ, Pedersen MB. 1995. Soil quality criteria for selected inorganic compounds. Working Report 48. Silkeborg, Denmark: Environmental Protection Agency.

Scott- Fordsmand J, Pedersen MB, Jensen J. 1996. Setting a soil quality criterion. *TEN* 3:20–24.

Smit and Van Gestel CAM. 1998. Effects of soil type, prepercolation, and ageing on bioaccumulation and toxicity of zinc for the springtail *Folsomia candida*. *Environ Toxicol Chem* 7:1132–1141.

Smith P, Hart ADM. 1994. Summary document for 24-27 May Organization for Economic Cooperation and Development workshop: comparison of ecological hazard/risk assessment schemes. Worplesdon, UK: Central Science Laboratory, Ministry of Agriculture, Food, and Fish.

Smolders E, Lambregts RM, Mclaughlin MJ, Tiller KG. 1998. Effect of soil solution chloride on cadmium availability to swiss chard. *J Environ Quality* 27:426–431.

Stephenson GL, Solomon KR, Hale B, Greenberg BM, Scroggins RP. 1996. Development of suitable test methods for evaluating the toxicity of contaminated soils to a battery of plant species relevant to soil environments in Canada. In Dwyer FJ, Doane TR, Hinman ML, editors. Environmental toxicology and risk assessment: modeling and risk assessment. Vol. 6, STP 1317. Philadelphia PA: American Society for Testing and Materials. p 474–489T.

Torstensson L, Pell M, Stenberg B. 1998. Need of a strategy for evaluation of arable soil quality. *Ambio* 27: 4–8.

[USEPA] U. S. Environmental Protection Agency. 1986. Ecological effects test guidelines, seedling emergence tier II. OPPTS 850.4225, 712-C-96-363. Washington DC: USEPA

[USFDA] U. S. Food and Drug Administration. 1987a. Environmental assessment technical book on seedling growth. Technical Assistance Document 4.07. Washington DC: USFDA.

[USFDA] U.S. Food and Drug Administration. 1987b. Seed germination and root elongation. Technical Assistance Document 4.06. Center for Food Safety and Applied Nutrition, Center for Veterinary Medicine, Washington DC: USFDA.

van Beelen P, Doelman P. 1996. Significance and application of microbial toxicity test in assessing ecotoxicological risks of contaminants in soil and sediment. RIVM Research for Man and Environment Report. No. 719102051. Bilthoven, The Netherlands: RIVM.

Van Gronsveld J, Colpaert JV, Van Tichelen KK. 1996. Reclamation of a bare industrial area contaminated by non-ferrous metals: physico-chemical and biological evaluation of the durability of soil treatment and revegation. *Environ Pollut* 94:131–140.

Van Gronsveld J, Van Assche F, Clijsters H. 1995. Reclamation of a bare industrial area contaminated by non-ferrous metals: in situ metal immobilization and revegatation. *Environ Pollut* 87:51–59.

Van Straalen NM. 1996. Critical body concentrations: their use in bioindication. In: Van Straalen, NM, Krivolutsky DA, editors. Bioindicator systems for soil pollution. NATO ASI, Series 2: Environment 16:5-16: Dordrecht, The Netherlands: Kluwer.

Van Straalen NM, Løkke H, editors. 1997. Ecological risk assessment of contaminants in soil. London UK: Chapman and Hall. 333 p.

Van Straalen NM, Schobben, JM, De Goede, RGM. 1989. Population consequences of cadmium toxicity in soil microarthropods. *Ecotoxicol Environ Safe* 17:190–204.

van Tilborg WJM, Van Assche F. 1995. Integrated criteria document: zinc; industry addendum. Rozendaal, The Netherlands: International Zinc Association. 50 p.